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For	:	ANNEXIN PROTEINS AND AUTOANTIBODIES AS SERUM MARKERS FOR CANCER	

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

INFORMATION DISCLOSURE STATEMENT

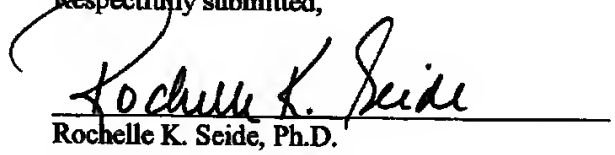
Sir:

Pursuant to 37 C.F.R. §§1.97 and 1.98, applicants respectfully request that the documents listed on the accompanying PTO 1449 be considered by the Examiner and made of record in the above-referenced application.

Identification of the listed documents is not to be construed as an admission of the applicants or attorneys for applicants that such citations are available as "prior art" against the subject application. If the Examiner applies the documents as prior art against any claim in the application and applicants determine that the cited documents do not constitute "prior art" under United States law, applicants reserve the right to present to the Office the relevant facts and law regarding the appropriate status of the documents. Applicants further reserve the right to take appropriate action to establish the patentability of the disclosed invention over the listed documents, should the documents be applied against the claims of the present application.

Applicants believe that there is no fee required for this submission. However, if any fee is required, or if any overpayment has been made, the Commissioner is hereby authorized to charge any fees, or credit or any overpayments made, to Deposit Account 01-2300.

Respectfully submitted,



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U.S. PATENT DOCUMENTS

Examiner Initial	Document Number	Date MM-YYYY	Name	Class	Sub-class	Filing Date If Appropriate
	US-					
	US-					
	US-					

FOREIGN PATENT DOCUMENTS

	Document Number	Date MM-YYYY	Country	Class	Sub-class	Translation Yes	Translation No

OTHER DOCUMENTS (Including Author, Title, Date, Pertinent Pages, Etc.)

		Brichory et al., An immune response manifested by the common occurrence of annexins I and II autoantibodies and high circulating levels of IL-6 in lung cancer. *PNAS*, Vol. 99 no. 17, August 14, 2003.
		Cervical cancer vaccine success, *BBC News*, March 14, 2006 16:11.

Examiner	Date Considered

EXAMINER: Initial if citation considered, whether or not citation is in conformance with MPEP § 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to the applicant.

An immune response manifested by the common occurrence of annexins I and II autoantibodies and high circulating levels of IL-6 in lung cancer

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The identification of circulating tumor antigens or their related autoantibodies provides a means for early cancer diagnosis as well as leads for therapy. The purpose of this study was to identify proteins that commonly induce a humoral response in lung cancer by using a proteomic approach and to investigate biological processes that may be associated with the development of autoantibodies. Aliquots of solubilized proteins from a lung adenocarcinoma cell line (A549) and from lung tumors were subjected to two-dimensional PAGE, followed by Western blot analysis in which individual sera were tested for primary antibodies. Sera from 54 newly diagnosed patients with lung cancer and 60 patients with other cancers and from 61 noncancer controls were analyzed. Sera from 60% of patients with lung adenocarcinoma and 33% of patients with squamous cell lung carcinoma but none of the noncancer controls exhibited IgG-based reactivity against proteins identified as glycosylated annexins I and/or II. Immunohistochemical analysis showed that annexin I was expressed diffusely in neoplastic cells in lung tumor tissues, whereas annexin II was predominant at the cell surface. Interestingly, IL-6 levels were significantly higher in sera of antibody-positive lung cancer patients compared with antibody-negative patients and controls. We conclude that an immune response manifested by annexins I and II autoantibodies occurs commonly in lung cancer and is associated with high circulating levels of an inflammatory cytokine. The proteomic approach we have implemented has utility for the development of serum-based assays for cancer diagnosis as we report in this paper on the discovery of antiannexins I and/or II in sera from patients with lung cancer.

There is increasing evidence for an immune response to cancer in humans, demonstrated in part by the identification of autoantibodies against a number of intracellular and surface antigens in patients with different tumor types (1–3). For example, somatic alterations in the p53 gene elicit a humoral response in 20–40% of affected patients (4). The detection of anti-p53 antibodies can predate the diagnosis of cancer (4). The majority of tumor-derived antigens that have been identified as eliciting a humoral response in lung cancer, as in other tumor types, are not the products of mutated genes. They include differentiation antigens and other proteins that are overexpressed in tumors (5). The oncogenic proteins L-Myc and C-Myc have been found to elicit autoantibodies in a small percentage of patients (1, 6). There is some evidence that occurrence of autoantibodies in lung cancer is of prognostic relevance (7–9). Remarkably, tumor regression has been demonstrated in some patients with small cell lung carcinoma and autoantibodies to oncofetal antigens (10, 11).

It is not clear why only a subset of patients with a tumor type develop a humoral response to a particular antigen. Immunogenicity may depend on the level of expression, posttranslational modification, or other types of processing of a protein, the extent of which may be variable among tumors of a similar type. Other factors that influence the immune response may include variability among individuals and tumors in major histocompatibility complex molecules. Cytokines, such as IL-1, IL-2, IL-6, tumor necrosis factor α (TNFα), or IFNγ, are also known to affect the immune response and may vary in concentration between tumors or in circulation (12, 13).

Although there is much interest in the identification of antigens that induce a cytolytic T cell response, the identification of panels of tumor antigens that elicit an antibody response may have utility in cancer screening or diagnosis or in establishing prognosis. Such antigens may also have utility in immunotherapy against the disease. We have implemented a proteomic approach for the identification of tumor antigens that elicit a humoral response. To this end, we have used two-dimensional PAGE (2-D PAGE) to simultaneously separate several thousand individual cellular proteins from tumor tissue or tumor cell lines. Separated proteins are transferred onto membranes. Sera from cancer patients are screened individually, for antibodies that react against separated proteins, by Western blot analysis. Proteins that react specifically with sera from cancer patients are identified by mass spectrometric analysis and/or amino acid sequencing. The goal of this study was to apply the proteomic approach to the identification of proteins that commonly elicit a humoral response in lung cancer.

Methods

Subjects. Tumor tissue and sera were obtained at the time of diagnosis after informed consent. The experimental protocol was approved by the University of Michigan Institutional Review Board. Sera from 54 lung cancer patients were analyzed. This group consisted of 29 males and 25 females with an age range of 40–82 years (median, 64.6 years). The diagnoses were adenocarcinoma (30 patients), squamous cell carcinoma (18 patients), small cell carcinoma (4 patients), and large cell carcinoma (2 patients), all histologically confirmed. Sera from 60 patients with other types of cancer (including 17 with esophageal, 11 with liver cancer, 14 with brain cancer, 11 with breast cancer, and 7 with melanoma) and from 61 other controls (including 51 healthy subjects and 10 subjects with chronic lung disease) were used as controls.

2-D PAGE and Western Blotting. After excision, the tumor tissue was frozen immediately at −80°C, after which an aliquot was lysed

Abbreviations: 2-D PAGE, two-dimensional PAGE; CRP, C reactive protein.

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in solubilization buffer (8 M urea/2% Nonidet P-40/2% carrier ampholytes, pH 4–8/2% 2-mercaptoethanol/10 mM PMSF) and stored at −80°C until use. Cultured A549 lung adenocarcinoma cells were harvested in 300 μl of solubilization buffer by using a cell scraper and stored at −80°C until use. Proteins derived from the extracts of either cultured cells or solid tumors were separated into two dimensions as described previously (14). The separated proteins were transferred onto a polyvinylidene fluoride membrane. Protein patterns in some gels were visualized directly by silver staining and, for some membranes, by Coomassie blue staining. For hybridization with serum, membranes were incubated with a blocking buffer consisting of Tris-buffered saline (TBS), 1.5% nonfat dry milk, and 0.01% Tween-20 for 2 h and then washed and incubated with serum at a 1:100 dilution for 1 h at room temperature. After three washes with washing buffer (TBS/0.01% Tween 20), the membranes were incubated with an anti-human IgG as secondary antibody at a 1:1,000 dilution (Amersham Pharmacia) for 30 min at room temperature, washed, and incubated briefly in ECL (enhanced chemiluminescence; Amersham Pharmacia)

Protein Identifications. For protein identification by mass spectrometry, 2-D gels were stained by using a modified silver-staining method and excised proteins were digested as described previously (15). A peptide mass profile was obtained by using a PerSeptive Biosystems matrix-assisted laser desorption ionization-TOF Voyager-DE Mass Spectrometer (Framingham, MA). The peptide masses obtained were used for database searches for protein identification.

Annexin Deglycosylation. Annexin I was purified from the A549 cell line by immunoaffinity chromatography. Briefly, the mouse mAb EH17a was purified by affinity chromatography with the mAb TrapGII kit and linked to the CNBr-activated Sepharose 4B (Amersham Pharmacia). The immunoaffinity column was used further to purify annexin I from the sonicated A549 cells. Purified annexin I was treated with endoglycosidase F according to the manufacturer's protocol (Roche Molecular Biochemicals, Indianapolis). Treated proteins were separated by SDS/PAGE and visualized by silver staining or transferred to a polyvinylidene fluoride membrane.

Immunohistochemistry. Immunohistochemistry for annexin I and II was performed by using an automated stainer (Ventana Medical Systems, Tucson, AZ). Annexin I antibody (ICN) was used at 1:100 dilution and annexin II (ICN) was used at 1:400 dilution. Formalin-fixed, paraffin-embedded sections of lung and lung tumors were stained by using the Ventana Basic DAB Detection Kit, which employs the avidin–biotin–complex method for the detection of primary annexin antibodies (16).

Cytokine and C Reactive Protein (CRP) ELISAs. The serum concentrations of IL-1β, IL-6, and TNFα were determined by using ELISA kits (Chemicon). The serum concentration of CRP was measured by indirect ELISA by using rabbit anti-human-CRP antibody and peroxidase-conjugated rabbit anti-human-CRP antibody (Dako). In all cases, a standard curve was constructed from standards provided by the suppliers.

Statistical Analysis. A comparison of cytokine and CRP serum levels was performed between patients with lung cancer (anti-annexin autoantibody-positive or antiannexin autoantibody-negative), healthy subjects, and patients with chronic lung disease. Results were expressed as means ± SEM. The statistical significance of differences between groups was determined by using the Wilcoxon two-sample test. Data were considered statistically significant if $P < 0.05$.

Table 1. Antiannexin I and II autoantibodies in subject sera

	Number of subjects	Annexin I autoantibody-positive	Annexin II autoantibody-positive
Lung cancer	54	18 (30%)	18 (33%)
Adenocarcinoma	30	12 (40%)	11 (37%)
Squamous cell carcinoma	18	3 (17%)	4 (22%)
Small cell carcinoma	4	1	2
Large cell carcinoma	2	0	1
Other types of cancer	60	5	0
Brain cancer	14	1	0
Breast cancer	13	1	0
Melanoma	7	0	0
Liver cancer	11	0	0
Esophageal cancer	17	4	0
Other controls	61	0	0
Healthy subjects	51	0	0
Chronic lung disease	10	0	0

IL-6 Treatment. A549 cells were incubated with or without IL-6 (10 ng/ml) for 24 h in DMEM without FCS. The culture supernatant subsequently was recovered and concentrated by using Centriprep 3 and Centricon 3 centrifugal filter units (Millipore). Cultured cells were washed three times with PBS, and the proteins bound to the cell membrane were EDTA-extracted for 30 min at 4°C in PBS supplemented with 1 mM EDTA and a mixture of protease inhibitors (Roche Molecular Biochemicals) and concentrated. Cultured cells were lysed by the addition of 300 μl of solubilization buffer and scraped. Protein concentrations were determined by means of the Bradford assay (Bio-Rad) before SDS electrophoresis and protein transfer to an Immobilon-P polyvinylidene fluoride membrane for Western blotting analysis with antiannexin I, antiannexin II, and anti-α-tubulin (Sigma) antibodies. Annexin I and II band intensities were normalized to α-tubulin intensities.

Results

Reactivity of Sera from Lung Cancer Patients with Annexins I and II. Sera obtained at the time of diagnosis from 54 patients with lung cancer including 30 with adenocarcinoma, from 60 patients with other types of cancer, and from 61 additional controls consisting of 51 healthy subjects and 10 subjects with chronic lung disease were investigated for the presence of antibodies to A549 adenocarcinoma cell line proteins (Table 1). Most lung cancer patient sera reacted against multiple proteins. The reactive proteins most commonly observed with lung cancer patient sera, but not with noncancer controls, consisted of two groups of contiguous proteins. The first group, with a pI between 7.6 and 8.2 and molecular mass of ≈36 kDa, was identified as annexin II by mass spectrometry (Figs. 1 and 2A). Annexin II reactivity was observed with sera from 18 of 54 (33%) patients with lung cancer, including sera from 11 of 30 (37%) adenocarcinoma patients (Table 1). A second group of contiguous protein spots, with a pI between 6.6 and 7.2 and molecular mass of 37 kDa, identified as annexin I by mass spectrometric analysis (Figs. 1 and 3A), was observed with sera from 16 of 54 (30%) lung cancer patients, including 12 of 30 (40%) with adenocarcinoma. Antibodies against both annexins I and II were observed with sera from 6 of 54 (11%) lung cancer patients, including 4 of 30 (13%) with adenocarcinoma and 2 of 18 (11%) with squamous cell carcinoma. Positive sera generally were reactive against annexins I and II at the highest serum dilution tested, which was 1:1,000. Polyvinylidene fluoride membranes prepared from the A549 adenocarcinoma cell line or from tumor tissue were hybridized with mAbs against annexin I or II. Protein spots that reacted with

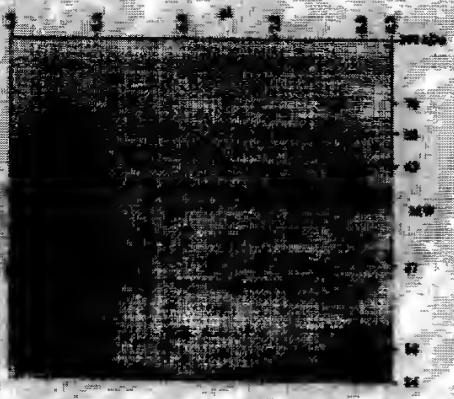
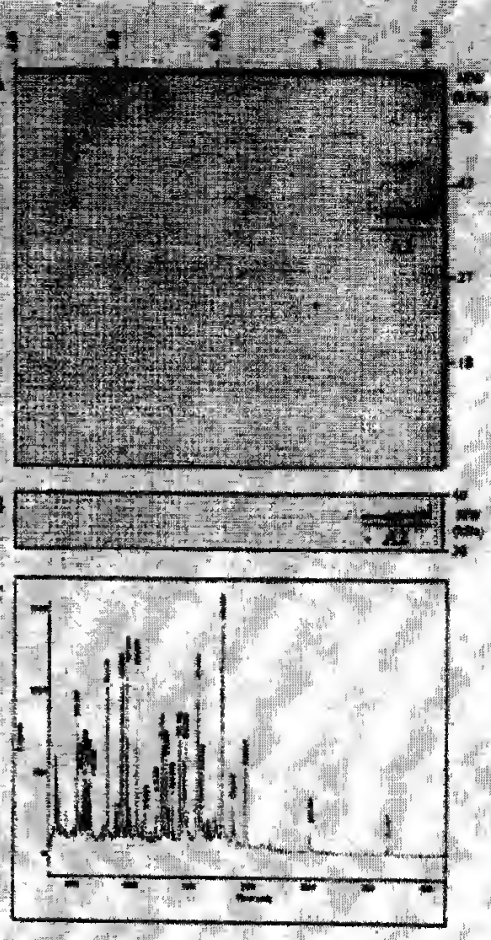


Fig. 1. Silver staining of A549 lung adenocarcinoma cell proteins separated by 2-D PAGE. Arrows point to the location of annexins I, II, IV, and V (A1, A2, A4, and A5) spots in the pattern.

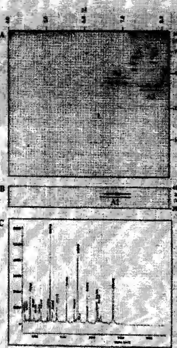


patient sera and that were identified as annexins I and II by mass spectrometry also reacted with the corresponding mAb (Figs. 2*B* and 3*B*). In total, 18 of 30 (60%) sera from patients with lung adenocarcinoma exhibited reactivity against annexin I and/or annexin II (Table 1). None of the sera exhibited immunoreactivity against other identified annexins in lung adenocarcinoma 2-D patterns, specifically annexins IV and V (Figs. 1, 2*A*, and 3*A*). Reactivity was not limited to patients with advanced-stage disease. Sera from 51% (19 of 37) of patients with stage I lung cancer contained autoantibodies to annexin I and/or II. Likewise, sera from 67% (6 of 9) of patients with stage II and 43% (3 of 7) of patients with stage III contained autoantibodies to annexin I and/or II. There was no correlation between smoking status and the occurrence of autoantibodies to annexin I and/or II in patients with lung cancer; 89% were smokers among patients with autoantibodies, and among patients without autoantibodies against annexins, 96% were smokers. For healthy subjects, 41% (21 of 51) were smokers. The occurrence of autoantibodies was not correlated with age of the patients (64.1 ± 1.5 for patients with sera containing antiannexin autoantibodies and 65.1 ± 1.7 for patients without antiannexin antibodies). Sera showed similar reactivity against annexins I and II in autologous tumor protein blots and in blots prepared from normal lung tissue, and in A549-derived blots (data not shown). Sera from lung cancer patients that exhibited IgG-based reactivity against annexin I and/or II exhibited reactivity that was specific to IgG1 among the IgG subtypes examined (IgG1–4) and also exhibited IgM-based reactivity (data not shown). None of the sera from other cancer types or from noncancer controls exhibited autoantibodies against annexin II. Annexin I autoantibodies were found in sera of 6 of 60 patients with other types of cancers, namely 4 of 17 with esophageal cancer, 1 of 14 with brain tumor, and 1 of 11 with breast cancer.

Expression of Annexins I and II in Tumor Tissue. Annexin expression in lung tumors was assessed by immunohistochemistry, using monoclonal anti-annexin I and II antibodies. We have analyzed 18 lung tumors, including 4 from patients with autoantibodies against annexin I, 4 with autoantibodies against annexin II, 4 with autoantibodies against Annexins I and II and 6 without anti-annexin autoantibodies. Annexin I was abundantly expressed in a diffuse manner in most adenocarcinomas (9 of 11) and squamous cell carcinomas (7 of 7) (Fig. 4). Intense annexin II immunoreactivity was also detected in a majority of tumors in a predominantly membranous pattern (8 of 11 adenocarcinomas and 5 of 7 squamous cell carcinomas) (Fig. 4). Lower expression levels for annexin II were observed in the other tumors (3

m/z	Peptide Sequence	Start	End
1834.8868	(K) WIHMTEK(S)	213	220
1893.3005	(R)QDIAFATQR(E)	69	77
1112.3978	(R)QDIAFAYQR(E)	69	77
2145.3482	(-)EFYEELCQDL	1	10
1443.6132	(K) M.YVVQQDTK(G)	334	336
1465.5491	(K) DYEPYDMLASIR(K)	254	265
1193.4358	(K) GLGTDEDSLIDICSSR(T)	138	152
1848.1168	(R) LELEGDMKKYPSAYGSVK(A)	11	28
1916.1988	(R) AEDGSVDYELIDQDARD(D)	188	196
2054.9708	(R) AAEDGSVDYELIDQDARD(D)	179	196

Fig. 2. (*A*) IgG1-based reactivity against annexin II protein in a Western blot of A549 proteins, using a lung cancer patient serum. (*B*) Close-up of a Western blot showing reactivity with antiannexin II mAb, confirming identity of the reactive protein shown in *A*. (*C*) Matrix-assisted laser desorption/ionization-time of flight spectra obtained from protein A2 after trypsin digestion and tryptic peptide sequences from annexin II matching with peaks obtained from the spectra.



m/z	Peptide Sequence	Start	End
908.0026	(K) ALYEAGER(R)	205	212
947.0257	(-) AMVSEFLK(Q)	1	8
963.3163	(-) AMVSEFLK(Q)	1	8
1065.1795	(K) ALYEAGERR(K)	205	213
1066.5705	(R) SEIDMNDIK(A)	304	312
1214.3765	(K) DITSDTSGDFR(N)	167	177
1254.3768	(K) LHQAMKGVGTR(H)	202	195
1263.3704	(K) TPAQFDADELR(A)	114	124
1371.5463	(K) CATSKPAFFAEK(L)	276	287
1501.7068	(K) GTDVNVFNTILTTR(S)	218	228
1620.3699	(K) TPAQFDADELRAAMK(G)	124	128
1653.6099	(R) KGTDVNVFNTILTTR(S)	214	228
1740.8627	(R) SEIDMNDIK(A)	289	304
1906.5776	(K) AAYLQETGKPLDETLKK(A)	82	98
2009.3984	(K) GDRSEDFGVNEDLADSDAR(A)	186	204
2058.4163	(K) GGPGSAVSPYPTFNPSSDVAALHK(A)	30	53

Fig. 3. (A) IgG-based reactivity against both annexin I and II proteins by using a patient serum. (B) Close-up of a Western blot showing reactivity of annexin I protein with antiannexin I mAb. (C) Mass spectrometry identification of annexin I after trypsin digestion of the protein A1.

adenocarcinomas and 2 squamous cell carcinomas), but the staining was also predominantly membranous. There were no appreciable differences in annexins I and II expression, by

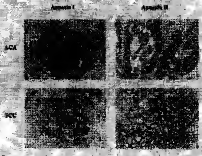


Fig. 4. Immunohistochemical analysis of annexin I and II expression in lung carcinomas. Representative immunoreactivity of annexins I and II in lung adenocarcinoma (ACA) and squamous cell carcinoma (SCC) (×300). Annexin I staining showed a mixture of nuclear, cytoplasmic, and membranous immunoreactivity, whereas annexin II staining showed immunoreactivity localized to the [illegible]plasmic membrane. T and S denote tumor and stroma tissue, respectively.

immunohistochemical analysis between autoantibody positive and negative lung cancer patients.

Role of Glycosylation in Annexin Antigenicity. We sought to determine whether annexin glycosylation contributed to immunogenicity. After purification, annexin I was subjected to N-deglycosylation. The resulting products were separated by SDS electrophoresis and analyzed by Western blotting (Fig. 5). N-deglycosylation by endoglycosidase F induced a basic shift of the protein without an apparent large molecular mass difference (Fig. 5B) compared with untreated annexin I (Fig. 5A). Two sera were tested that exhibited IgG-based immunoreactivity against annexin I. These sera did not react against endoglycosidase F-treated annexin but exhibited IgG-based immunoreactivity

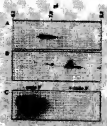


Fig. 5. (A) Western blot analysis of purified annexin I before enzymatic treatment. (B) Western blot analysis of purified annexin I after endoglycosidase F treatment, showing a basic shift in annexin I migration. A and B were 2-D PAGE Western blot hybridized with antiannexin I mAb. (C) One-dimensional SDS/PAGE Western blot of purified annexin I, hybridized with a serum from an annexin I antibody-positive lung cancer patient. Hybridization was observed before (− endo F) but not after (+ endo F) N-deglycosylation by endoglycosidase F.

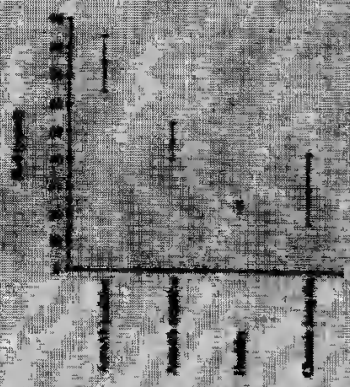


Fig. 6. Serum IL-6 levels in annexin autoantibody (anti-Ab)-positive and anti-Ab-negative lung cancer patients, in healthy subjects, and in patients with chronic lung disease.

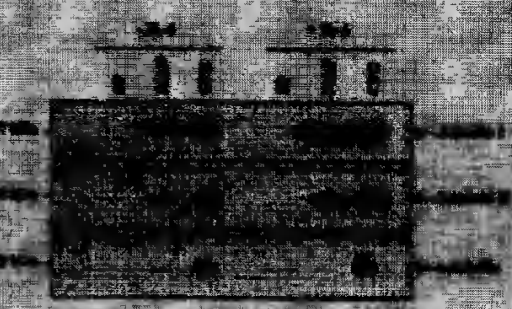


Fig. 7. Western blot analysis of proteins from the A549 cells, untreated or untreated with IL-6, separated by one-dimensional SDS-PAGE, and subsequently individually with either anti-annexin I, or anti-annexin II monoclonal antibodies. An increase in the membrane-associated fraction (MAF) was observed after IL-6 treatment. SF and CPF denote secreted protein and cytosolic protein extract fractions, respectively.

against annexin I, which we had observed already by 2-D PAGE Western blot with this patient's serum (Figs. 3*A* and 5*C*).

Assays of IL-1β, IL-6, TNFα, and CRP in Subject Sera. High serum levels of IL-6 have been reported in some patients with lung cancer (12, 13). We therefore determined whether patients that exhibited immunoreactivity against annexins I and II exhibited different serum levels of IL-1β, IL-6, TNFα, and CRP from noncancerous patients and controls. Sera from a total of 40 patients with lung cancer (20 with antiannexin I and/or II autoantibodies and 20 without antiannexin autoantibodies), from 39 healthy subjects, and from 10 patients with chronic lung disease were investigated. Compared with healthy subjects, patients with lung cancer had significantly higher serum levels of IL-6 (healthy subjects, 23.14 ± 1.38 pg/ml; patients, 60.23 ± 6.27 pg/ml; $P = 0.003$) (Fig. 6) and higher CRP levels (healthy subjects, 124.19 ± 26.64 ng/ml; patients, 529.33 ± 102.47 ng/ml; $P = 0.001$). In addition, patients with autoantibodies against annexin I and/or II had significantly higher IL-6 serum levels (74.25 ± 9.75 pg/ml) than patients without antiannexin autoantibodies (46.82 ± 6.58 pg/ml; $P = 0.029$) (Fig. 6). No statistically significant differences in IL-1β and TNFα serum levels were observed between the different groups.

Effect of IL-6 Treatment on the Expression of Annexins I and II. IL-6 has been identified as a major cytokine expressed by tumor-infiltrating cells in lung cancer (17). We therefore examined the effect of IL-6 treatment on the expression of annexins I and II in lung cancer cells. A549 cells were treated with IL-6 for 24 h, and the cytosolic, membrane-associated, and secreted protein fractions were analyzed by Western blotting with antiannexin I and II mAbs. IL-6 treatment resulted in an increase in exocytosis-associated annexins I and II (3.2- and 2.3-fold increase in band intensity, respectively; Fig. 7) and in faintly detectable annexin I and II bands in the secreted protein fraction (Fig. 7). No appreciable change was observed in annexin levels in the cytosolic fraction after IL-6 treatment.

Discussion

In this study, sera from more than half of the patients with lung cancer exhibited IgG1 and IgM autoantibodies to annexin I and/or II. Annexin II autoantibodies were found only in lung cancer patients in our series, whereas annexin I autoantibodies also were observed in a few patients with other cancers. The annexins belong to a family of multifunctional, calcium-dependent, phospholipid-binding proteins (18). Prior studies have shown that, in the lung, annexins I and II were expressed in cilia and pleural mesothelial cells but not in Clara cells (19). Annexin I but not annexin II was found to be expressed in epithelium. In addition, annexin II was expressed in type I and II alveolar cells where no expression of annexin I was observed. We have shown that annexins I and II were highly expressed in lung cancer cells. Annexin I is a 37-kDa protein that has been implicated in glucocorticoid-induced inhibition of cell growth (20, 21). Annexin II is a 36-kDa protein that occurs in a monomeric form or as a tetramer, associated with the annexin II light chain (p11), which is a member of the S100 family (22). Annexin II has been implicated in cell–cell adhesion and in plasminogen activation and may function as a cell surface receptor (23). Annexin II tetramers have been shown to interact with procathepsin B on the surface of tumor cells and may be involved in extracellular proteolysis, facilitating tumor invasion and metastasis (24). Interestingly, annexin I is a target of autoantibodies in autoimmune diseases such as systemic lupus erythematosus (25) and rheumatoid arthritis (26). Annexin II, specifically, has not been implicated previously as a target of autoantibodies in any disorders.

Annexins are known to undergo posttranslational modification including glycosylation (27). Annexins I and II are both phosphorylated by various kinases (28). In our study, immunoreactivity against annexin I was found to be dependent on N-glycosylation. At potential N-linked glycosylation site is present at positions 42 and 61 from the N terminus of annexins I and II, respectively (29, 30). Glycosylation may contribute to protein stability and may enhance signal transduction (27). Although immunoreactivity was dependent on N-glycosylation, there was no indication that such glycosylation or any other posttranslational modification associated with immunoreactivity was restricted to cancer cells or to cancer-sensitive patients, because sera from immunoreactive patients also reacted with annexins I and II from normal lung. This suggests that an elevated immune response and/or altered levels and cellular distribution of annexins I and II in lung cancer patients account for the development of antiannexin autoantibodies. Nevertheless, our data clearly indicate that identification of some antigenic proteins, as in the case of glycosylated annexins, necessitates the screening of proteins in their modified state. This is difficult to achieve with other screening approaches that rely on recombinant proteins that may lack antigenic and other modifications necessary for reactivity with autoantibodies (31).

An increased level of serum IL-6 in some patients with lung cancer has been reported previously and shown to be part of an inflammatory response (17, 32). Also, cytokine-expression analyses of tumor-infiltrating cells in non-small-cell lung cancer have shown that IL-6 was the predominant cytokine expressed (17). We have shown that patients that exhibited autoantibodies to annexins I and II had significantly higher IL-6 serum levels than any of our comparison groups, most importantly lung cancer patients without demonstrable antiannexin autoantibodies. This finding suggests that host factors such as cytokines may affect the immune response to potentially antigenic proteins. We also have shown that IL-6 treatment of A549 resulted in an increase in membrane-bound annexin I and II, supporting a prior finding of an IL-6-stimulated translocation of annexin I to the cell surface (33). An increase in membrane-associated annexins I and II may enhance immunogenicity.

A prerequisite for an immune response against a cellular protein is its presentation as an antigen. Interestingly, it has been shown previously in a melanoma cell line that annexin II-derived peptides are bound to MHC class II molecules (34). Expression of MHC molecules in lung cancer cells also may be induced as a result of cytokine secretion by tumor-infiltrating cells (35). Thus, inflammatory cytokine(s) may contribute to an increased expression of MHC molecules and to annexin I and II translocation to the cell surface, resulting in a humoral immune response.

Although in our study autoantibodies to annexin I were observed in some patients with tumors other than lung cancer, annexin II autoantibodies were restricted to lung cancer patients. Prior studies have demonstrated increased expression of annexin I and/or II in different tumor types (36–40). Thus, the extent to which annexin II autoantibodies may occur in tumor types other than lung cancer requires further investigation, in particular, in cancer types in which increased annexin II has been observed previously, as in the case of glioblastoma multiforme (39), pancreatic cancer (40), and acute promyelocytic leukemia (41). Interestingly, it has been shown recently that expression of annexin I is lost in esophageal squamous cell cancer (42). In our study, we have shown that some sera from patients with esophageal cancer (4 of 17) exhibited autoantibodies against annexin I. Most of these esophageal cancers were adenocarcinomas (15 of 17), and the four immunoreactive sera against annexin I were from patients with esophageal adenocarcinomas.

Our findings led us to propose a mechanism for the development of autoantibodies against certain proteins in cancer, whereby host factors such as cytokines may affect the level and cellular distribution of a potential antigen, the presentation of specific polypeptides, and also modulate the immune response in other ways. Given the high frequency with which autoantibodies to certain proteins may occur in cancer, as we have demonstrated for autoantibodies to annexins I an II in lung cancer, assays for panels of such circulating antibodies and/or their corresponding antigens may have clinical utility.

The mouse monoclonal antiannexin I antibody, EH17a, developed by J. D. Ernst, was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa, Department of Biological Sciences, Iowa City. We thank Drs. M. B. Orringer, M. D. Iannettoni, and D. A. Arenberg for their assistance in sample procurement. This work was supported in part by research funding from the National Cancer Institute Early Detection Research Network Program and by a fellowship for Franck Brichory from the French Association for Cancer Research.

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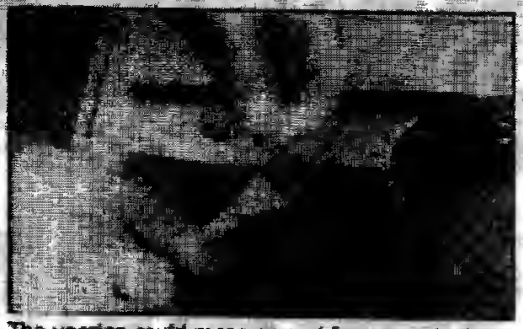
Cervical cancer vaccine success



The vaccine could mean an end for smear tests

A vaccine to protect women from one of the biggest cancer killers could be available within five years.

Early clinical trials of a vaccine for cervical cancer have shown that it is 100% effective. It also protects against genital warts.

The breakthrough could help to save thousands of lives each year.

Cervical cancer is the second most common cancer in women under 35 in the UK. It claims 1,300 lives each year.

> **This is really the holy grail of cancer research. It is very exciting**
>
> **Dr Anne Szarewski, Cancer Research UK**

The vaccine works by triggering the body's immune system to attack the human papiloma virus (HPV), which has been linked to almost all cases of cervical cancer.

Further trials

Merck Sharp & Dohme, the company behind the vaccine, said further studies are needed but that it could be available in a few years.

The vaccine would be given to teenage girls. It would only work in females who have not yet become sexually active.

This is because HPV is transmitted through sexual intercourse.

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UK
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N Ireland
Scotland
Wales
Politics
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The vaccine fights four of the most common strains of HPV, including a strain that causes genital warts.

Early trials on almost 2,400 women between the ages of 16 and 23 in the United States have shown that it reduced the incidence of HPV by 100% after one year.

Merck Sharp & Dohme is now recruiting 6,000 women worldwide to take part in phase three trials.

Prof Jenkins is heading the Nottingham trial

These will include 250 women at centres in Glasgow, London and Nottingham.

If the trials are successful the company will then be able to apply for a licence to manufacture and sell the vaccine.

A company spokeswoman told BBC News Online: "Recruitment is currently under way in three centres in the UK. If these phase three trials are successful then we may have a vaccine within several years."

Professor David Jenkins, who will lead the Nottingham study, said: "What we're trying now is to see if this can be rolled out into real life and into preventing cervical cancer. But it will take five years at least."

Breakthrough

Dr Anne Szarewksi, a clinical consultant at Cancer Research UK, described the results as "very exciting".

"These results look very, very good. People have been trying to get a vaccine for years and years," she told BBC News Online.

"This is really the holy grail of cancer research. It is very exciting."

But Dr Szarewski warned that the vaccine would not help women who have already become sexually active.

"This vaccine would have to be given to teenage or young girls who haven't become sexually active yet.

"An entire generation who have already become sexually active would not benefit."

But she added: "Once it does become available to a new generation of women then I see a situation where they will no longer have to have smear tests."

HPVs are a group of more than 80 different types of virus. They can be transmitted through sexual intercourse.

It is estimated that up to 15% of women aged 20 to 30 women and up to 6% of women over 40 carry the virus. The majority do not go on to develop cancer.

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